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RECOMMENDED STANDARD PROCEDURES FOR LABORATORY DIAGNOSIS OF TUBERCULOSIS IN ANIMALS

Agricultural Research Service
UNITED STATES DEPARTMENT OF AGRICULTURE

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Prepared by

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PREFACE

This publication has been prepared, under the State-Federal Cooperative Tuberculosis Eradication Program, to establish recommended standards for laboratory examination of animal tissue for the presence of organisms of the genus Mycobacterium.

The standards were prepared by two special committees of the National Conference of Veterinary Laboratory Diagnosticians, adopted by the United States Livestock Sanitary Association[1] in Phoenix, Ariz., October 1967, and approved by the Animal Health Division, U.S. Department of Agriculture.

Comments or suggestions for changes in the standards should be submitted to the Director of the Animal Health Division, Agricultural Research Service, U.S. Department of Agriculture, Federal Center Building, Hyattsville, Md. 20782.

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[1] Renamed: United States Animal Health Association.

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RECOMMENDED STANDARD PROCEDURES FOR LABORATORY DIAGNOSIS OF TUBERCULOSIS IN ANIMALS

INTRODUCTION

Approximately one-half of the lesions suspected of being tuberculous have been found to be due to some other cause (3,4,8).[1] Therefore, histopathologic examination of suspected lesions is necessary to confirm the diagnosis of tuberculosis.

At this stage of the tuberculosis eradication program, it is desirable to have a standard method for the diagnosis of tuberculosis. This method should include the gross inspection of tissues plus histopathologic and bacteriologic examinations. The examinations for tissue changes and acid-fast bacteria should be conducted at the same laboratory so that a complete evaluation can be made.

Acid-fast bacilli have been cultured from lesions caused by other etiologic agents, also from apparently normal tissues, water sediments, paraffin, and gelatin (2,8,10). Therefore, it is important that adequate tissue submissions with good histories be received at the laboratory. Laboratory procedures should be carried out using proper safety measures (see Appendix A) and controls, such as standardized reagents and slides prepared from known infected and noninfected tissue sections.

STANDARD PROCEDURES FOR THE HISTOPATHOLOGIC DIAGNOSIS OF TUBERCULOSIS IN ANIMALS

Collection and Shipment of Specimens

Specimens collected for histopathologic examination should be selected to demonstrate lesions and adjacent normal tissue.

The tissues should be sliced approximately 1 centimeter thick so that they will fix in the formalin solution. Selected tissues should be placed in specimen jars containing 10 percent neutral formalin. (Ten percent formalin is a 1:10 dilution of commercial 40 percent formalin in saline buffered to pH7.) The ratio of tissue to formalin should not exceed one part tissue to 10 parts formalin.

Animal Health Division form 6-35 or 10-1 should be filled out and enclosed in the shipping carton with the specimen jar. Back tag numbers, ear tag numbers, sex, gross and dressed weight, postmortem findings, and any other pertinent information should be recorded on the submission form.

[1] Underscored numbers in parentheses refer to Literature Cited, p. 16.

Staining Procedures

Separate tissue sections should be stained to demonstrate cellular detail and acid-fast bacteria. Hematoxylin and eosin (H & E) stain is routinely used to demonstrate cellular detail. The following H & E staining procedure is suggested:

Hematoxylin and Eosin Stain

1. Cut tissue in blocks approximately 3/4 x 3/4 x 1/4 inch thick and place in tissue holder buttons.
2. Place in 10 percent neutral formalin.
3. Process overnight through Autotechnicon (see Appendix D) using the following schedule:

Ingredients	Hours	Ingredients	Hours
Dehydrant[1]	1	Dehydrant	1
Dehydrant	2	Dehydrant	1
Dehydrant	1	Dehydrant	1
Dehydrant	1	Clearing agent[1]	1 1/2
Dehydrant	1	Paraplast[1]	2
Dehydrant	1	Paraplast	2 1/2

[1] See Appendix D.

4. While the tissue is in the final paraplast solution, draw a 25-lb. negative pressure and hold for several minutes to insure adequate infiltration.
5. Embed tissue in paraplast using a Thermoelectric Centre and Tissue Tek embedding rings. (See Appendix D.)
6. Cut embedded tissue blocks at 6 microns on rotary microtome.
7. Mount tissue sections on glass slides.
8. Place slides in slide drier at 80° - 85° C. for 10 to 15 minutes.
9. Stain tissue with H & E in the Autotechnicon using the following scheduled:

Ingredient	Minutes	Ingredient	Minutes
Xylene	10	Tap water	2 1/2
100 percent alcohol	4	Potassium acetate	2
95 percent alcohol	3	Alcoholic eosin Y[1]	4
70 percent alcohol	3	95 percent alcohol	2 1/2
Tap water	2	100 percent alchol	4
Harris's hematoxylin[1]	1 1/2	Xylene.	

[1] See Appendix B.

10. Coverslip using a synthetic resin permanent mounting media. (See Appendix D.) New Fuchsin stain is used to demonstrate acid-fast bacilli.

New Fuchsin Staining

Acid-fast bacilli are stained with New Fuchsin by the following procedure: Follow steps 1-8 as described for H & E staining, then follow steps 9-19.

9. Deparaffinize through three changes of xylene, dehydrate through two changes 100 percent alcohol.[2] Dry. Dip in Collodion solution to make sure tissue does not come off the slide. Dry.
10. Dip in distilled water.
11. Stain in New Fuchsin. (See Appendixes B and D.)
12. Dip in saturated solution of lithium carbonate.
13. Decolorize in acetic alcohol (see Appendix B) until no more color runs out.
14. Rinse in running water.
15. Counterstain in methylene blue (see Appendix B) for 1 to 2 minutes.
16. Rinse in running tap water.
17. Pass through three changes of 99 percent alcohol.
18. Pass through four changes of xylene.
19. Mount in Permount.

Fluorescent Staining

When tissue changes suggestive of tuberculosis are present, and no acid-fast bacilli are demonstrated by routine stains, fluorescent stains should be used (1). The following procedure for Auramine O staining[3] is recommended:

1. Deparaffinize tissues for 5 minutes each in three changes of xylene.
2. Place the slides in each of the following solutions successively for approximately 5 minutes: three changes of absolute alcohol and one change each of 95 percent alcohol, 80 percent alcohol, and 70 percent alcohol.
3. Stain for 10 minutes in Auramine O staining solution prepared as follows:

Ingredients	Amount
Auramine O (C.I. 41000, see Appendix D) . . . . . grams . .	3
Liquefield phenol . . . . . milliliters . .	32
Glycerin . . . . . do	70
Distilled water. . . . . do	900

4. Decolorize with one quick dip in 20 percent sulfuric acid.
5. Dip quickly in 80 percent alcohol.
6. Place in 10 percent ferric chloride for 5 minutes.
7. Place in 95 percent alcohol for 30 seconds, and allow the slides to air dry.
8. Coverslip[4] with mounting media (see "Related Procedures") for fluorescent microscopy.

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[2] In this paper, the term "alcohol" always refers to ethyl alchol.

[3] Howell, D. G. Selected Studies of the Fluorescent Staining of Acid-Fast Bacilli in Bovine Tissues with Auramine O. Master's Thesis, Iowa State University of Science and Technology, Ames, Iowa. 1966.

[4] Special coverslips are recommended. (Corning coverglass No. 1.5 thickness, Corning Glass Works, Corning, N.Y.).

Mounting Media

Materials	Amount
Styron 666 (see Appendix D) . . . grams . .	10
Dibutyl phthalate . . . . . milliliters . .	5
Xylene . . . . . do	35

The Styron must be thoroughly dried in a drying oven at 750° C. for 2 hours before use. The mixture is allowed to stand overnight (10 to 12 hours). More xylene may be added if the consistency is too thick.

Decalcification Procedure

Granulomatous lesions are often calcified and must be decalcified before tissue sections can be cut. The following procedure may be used:

1. Cut tissue in blocks approximately 3/4 x 3/4 x 1/4 inch thick and place in tissue holder buttons.
2. Place tissue buttons in decalcification solution (Appendix D) overnight.
3. Wash in running water for at least 3 hours.
4. Process like ordinary tissue, except, when staining decalcified tissue with New Fuchsin, add 0.1 percent borax to the methylene blue.

Cutting Frozen Tissue Sections

When prompt results are desired, as when the case is retained and the carcass is held at the processing plant pending laboratory results, a rapid decalcification process may be used prior to cutting frozen sections (5, 9). In this process, the tissue buttons are placed in rapid decalcification solution (Appendix D) for approximately 2 to 3 hours. They are then washed a few minutes in running water, frozen and cut on a cryostat (freezing microtome), and stained by the usual method. This process has the advantage of being fast; it does not interfere with staining acid-fast bacilli, but also does not bring out all the cellular detail obtained by conventional methods.

Controls for Stains and Procedures

Controls should be processed to insure the reliability of the above methods. Procedures should be routinely checked (at least once a week) by processing known normal and mycobacteria infected tissues.

Reporting

Lesions typical of tuberculosis in which acid-fast bacilli are demonstrated should be considered tuberculous. Reports should indicate one of the following categories:

Compatible--mycobacteria and tuberculous lesions present.

Suggestive--granulomatous lesions present, but neither mycobacteria nor other etiologic agents found.

Not suggestive--neither mycobacteria nor granulomatous lesions found.

When other etiological agents are demonstrated, cases should be reported.

STANDARD PROCEDURES FOR THE ISOLATION AND TYPING OF MYCOBACTERIA

Selection and Shipment of Specimens for Mycobacterial Examination

The proper collection and shipment of tuberculosis specimens is vital to the successful isolation of mycobacteria.[5] The affected tissue and adjacent lymphoid tissue should be submitted.

1. Specimens for routine isolation of mycobacteria. In general, the affected tissue and adjacent lymphoid tissues should be submitted. If no lesions are observed but there is justification for the mycobacteriologic examination, lymph nodes should be selected to represent the head, thorax, and abdomen.

The tissues should be placed in a 4-ounce specimen bottle which has previously been filled with water to dissolve the chloramine T. A minimum of 10 grams of fat-free tissue is required. When infection is discovered by incising a lymph node, that node and one adjacent to it (unincised) should be submitted.

The bottles containing specimens should be placed in a refrigerated polystyrene container and returned to the laboratory.

Isolation of Mycobacteria

Reporting New Cases

An accession number should be assigned to the specimens of each case upon arrival. This number should accompany the case during all laboratory processes and reporting.

Preliminary Treatment of Specimens

Tissue specimens are rinsed in a dilute solution of sodium hypochlorite[6] (1,000 parts per million) as soon as possible after receipt. All fat is removed from tissues using separate sterile scissors and forceps for each specimen. Avoid incising uncut lymph nodes.

The treated tissues are submerged in fresh, 1,000 p.p.m. sodium hypochlorite solution and allowed to stand undisturbed overnight at 4° C. The following day the tissues are washed twice in fresh 1,000 p.p.m. sodium hypochlorite solution.

Grinding and Digesting

The washed tissues are placed in sterile petri dishes and transferred to a biological safety cabinet where they are dissected with sterile scissors and forceps. The cut surfaces are examined for lesions.

The central area from each tissue is transferred to a blender jar. Lesions are included with adjacent tissue. Fifty milliliters of nutrient broth,

[5] See Appendix A.

[6] Prepared by mixing 76 ml. of 5.25 percent NaOCl with 3,924 ml. of tap water.

containing 0.4 percent phenol red indicator, is added to the blender jar. The mixture is blended for 2 minutes. A Teflon magnetic stirring bar and 100 ml. of papain are added to the blended tissues. Enough 4.0 percent NaOH is added to change the phenol red indicator from yellow to red.

The outside of the blender jar is disinfected with 2.0 percent Amphyl solution and removed from the safety cabinet.

Digestion of the tissues is enhanced by agitating for 1 hour on a magnetic mixer. The temperature of the digestion mixture should be maintained at 37° C. in a waterbath.

Concentration

The blender jar is then returned to the biological safety cabinet. Ten milliliters of pentane is added to the contents. (Pentane is flammable and all burners in the cabinet should be off.) The contents are mixed by two or three vigorous shakes and allowed to stand undisturbed for 30 minutes.

The mycobacteria that were released from the tissue during digestion are oriented in the pentane-water interface. The interface between the pentane layer and the aqueous layer is removed with a sterile 10-ml. pipette and filtered through a double layer of sterile unbleached muslin. The muslin may be supported by a glass funnel. The filtrate is collected in a sterile test tube, 20 x 125 mm.

The tube and contents are centrifuged at 1,552 RCF[7] for 20 minutes inside a screw cap safety centrifuge capsule. The sediment is deposited in the bottom of the tube, and a pellicle is formed on top of the liquid during centrifugation. The liquid separating the sediment and pellicle is removed with a pipette and autoclaved.

One milliliter of nutrient broth is added to the centrifuge tube, which is shaken vigorously, producing a homogenous mixture of sediment, pellicle, and broth. This mixture is used to inoculate media and animals.

Culture Mediums

Each of eight tubes of culture mediums is inoculated with four drops of inoculum delivered from a pasteur pipette. The mediums inoculated for each tube (see Appendix C) are as follows: Stonebrinks (two slants; one enriched with sodium pyruvate and one not enriched with sodium pyruvate); Herrolds (two slants; one with glycerol and malachite green and one without glycerol or malachite green): and Lowenstein-Jensen (two slants; one with glycerol and one without glycerol).

The tubes containing inoculated slants are disinfected and removed from the biological safety cabinet. Each tube is held so that the inoculum flows evenly over the surface of the medium. The tubes are placed in a rack so that they remain horizontal during overnight incubation of 37° C. This step insures uniform contact of the inoculum over the entire surface of each slant. The slants are returned to the vertical position for further incubation at 37° C.

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[7] RCF = relative centrifugal force = $\frac{4 \times (3.14)^2 \times r \times n^2}{32.2}$ where r = radius in feet and n = revolutions per second.

Example.--If a centrifuge head that accommodates extended screw cap test tubes with an effective radius of 9 1/2 inches is operated at 2,400 r.p.m., then the RCF is 1,552.

The excess inoculum is mixed with an equal volume of 0.2 percent benzalkonium chloride to suppress the growth of contaminating microorganisms and refrigerated. If the inoculated medium is overgrown or hydrolyzed by contaminating microorganisms, a second set of mediums is inoculated with the benzalkonium chloride treated inoculum.

Incubation Period

The incubating culture mediums are examined each week for 12 weeks for the presence of mycobacterial colonies. If bacterial colonies resembling those of mycobacteria are found, a smear is made from each. The smear is stained by the Ziehl-Neelsen technique and observed under oil immersion for the presence of acid-fast bacilli. Each colony of acid-fast microorganisms is used to inoculate a single tube of Dubos broth containing Tween 80 (see Appendix D) and albumin. The broth is used at a later time for biochemical tests and to study cultural characteristics.

Typing of Mycobacteria

Observation of Primary Colonies

An understanding of the variations in colonial morphology resulting from changes in the medium or by subculturing is essential for an effective mycobacterial typing scheme. Isolated colonies are observed for morphology and pigmentation with the aid of a hand lens or other optical device.

Mycobacterium bovis.--Primary colonies of this species on Middlebrook's 7-H-10 medium usually appear colorless, flat, irregular, rough, and very dull by transmitted light. They appear flat and dry or resemble the convex, white, moist colony of M. avium and the anonymous mycobacteria on Herrold's medium. M. bovis isolants, subcultured on Lowenstein-Jensen medium, produce mammilate, colorless colonies (fig. 1). M. bovis colonies growing on Stonebrink medium usually appear as white, moist, convex colonies resembling those of M. avium.

Mycobacterium avium.--Primary colonies of this species usually appear moist, white, and convex on all four types of media.

Runyon Group III.--Same as M. avium.

Mycobacterium tuberculosis.--Primary colonies are very dry, rough, flat to raised, and white to buff with irregular edges when grown on Lowenstein-Jensen medium.

Cellular and colonial characteristics of several mycobacteria and their respective biochemical reactions are presented in table 1.

Biochemical and Cultural Procedures

Smears are made of all colonies resembling those of mycobacteria. The smears are stained with the Ziehl-Neelsen procedure described in Appendix B.

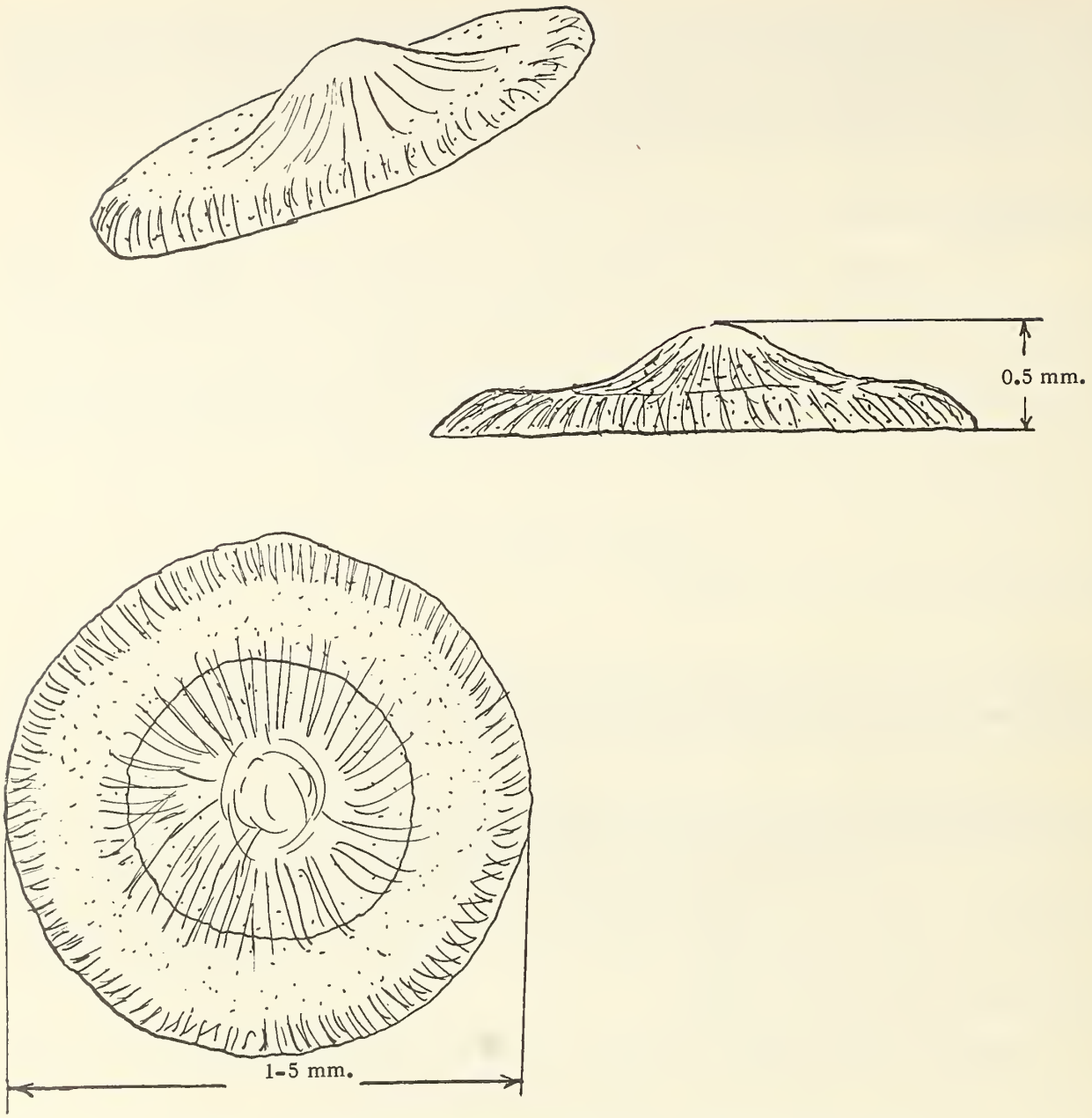

Figure 1.--Typical Mycobacterium bovis colony on Lowenstein-Jensen medium.

The remaining portion of each colony is transferred to Dubos broth (containing albumin and Tween 80) with a sterile wire loop. The broth is incubated for 1 week at 37° C. The purity of new growth is confirmed by smear and Ziehl-Neelsen staining. The incubation period is extended for another week if no growth appears.

Both fluid and solid media are inoculated with the pure Dubos broth culture for use in subsequent typing procedures.

Proskauer and Beck medium (6) is used to study four cultural characteristics in fluid medium as shown in the following tests and observations:

Granular-uniform growth characteristics. Cells of M. tuberculosis and M. bovis cultures have a granular (clumped) appearance in transmitted light.

Table 1.--Pertinent characteristics of three mycobacterial types and four unclassified groups

[Plus and minus signs indicate the presence or absence of the character named in the column heading. Growth character in P and B: G, granular growth; U, uniform homogenous growth. Colonial morphology: C, convex; D, dry; E, entire; F, flat; H, heaped; I, irregular; M, mammilate; Mst, moist; R, rough; S, smooth. Catalase activity: M, moderate; S, slight; St, strong; T, trace]

Mycobacterial type	Appearance time, in days, at--		Pigment production		Cording	Growth character in P and B medium	Growth in--		Neo-tetrazolium	Colonial morphology	Niacin test	Catalase activity	Tellurite reduction (3-day)	Animal pathogenicity[1]		
	37° C.	45° C.	In light	In dark			INH	TCH						Guinea pigs	Rabbits	Chickens
Mycobacterium bovis.....	13-20	([2])	-	-	+	G	-	-	-	M, I	-	T	-	Yes	Yes	No
M. avium.....	13-20	6-13	-	-	-	U to G	+	+	+	S, C, E, Mst	-	S	+	No	Yes	Yes
M. tuberculosis...	13-20	([2])	-	-	+	G	-	+	-	D,R,H,I	+	T	-	Yes	No	No
Runyon Group:																
I.....	13-20	([2])	+	-	-	U	+	+	+	S to R, C,Mst,E	-	M	-	---	---	---
II.....	13-20	([2])	+	-	-	U	+	+	+	S to R, C,Mst,E	-	M	-	---	---	---
III.....	13-20	6-13	-	-	-	U to G	+	+	+	S,C,Mst, E	-	S	-	---	---	---
IV.....	([3])	([4])	([4])	([4])	([4])	U to G	+	+	+	([4])	-	St	-	---	---	---

[1] Not applicable to Runyon Groups I to IV.
[2] No growth.
[3] Six or less.
[4] Variable.

All other mycobacteria usually have a uniform (homogenous dispersion of cells) appearance in transmitted light. Rough strains of M. avium and Runyon's anonymous mycobacteria often produce clumps of cells in a background of uniform cells (fig. 2).

Cording.--Cording is a term used to describe a parallel alinement of bacterial cells that results in a long serpentine or stringlike formation. M. tuberculosis and M. bovis produce cords consistently; however, other mycobacteria may also produce cording. The phenomenon is an indicator of virulence. A smear of the bacterial sediment in a Proskauer and Beck culture is stained by Ziehl-Neelsen technique and examined by oil immersion microscopy for the presence of cords and for cellular morphology (fig. 3).

Cellular morphology.--Cellular morphology may be studied on the same smear and the observations used to aid in determining the bacterial type. The morphology of several mycobacteria is presented in table 1.

Niacin.--The presence of niacin in a culture growing in Proskauer and Beck medium may be used to differentiate M. tuberculosis from all other mycobacteria (6). M. tuberculosis is a vigorous producer of niacin. M. fortuitum and M. marinum have also been reported as niacin producers.

One milliliter of 4.0 percent aniline in ethyl alcohol is added to the culture growing in Proskauer and Beck medium. This is followed by the addition of 1.0 ml. of aqueous cyanogen bromide--deadly poison. (See Appendix C, under niacin test reagents.) Yellow appearing within 5 minutes indicates the presence of niacin.

Biochemical sensitivity tests are conducted in the basal medium, which is Dubos broth with albumin and Tween 80 Iso-nicotinic acid hydrazide (INH) (see Appendix C) prepared in a final concentration of 10 μg. per ml. of basal

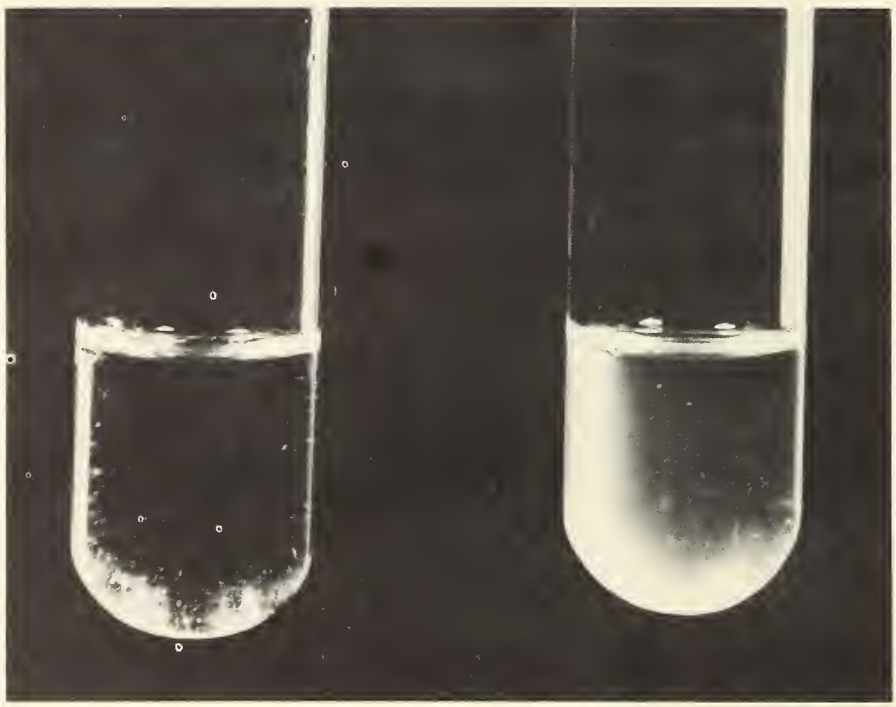

Figure 2.--Left, clumping of cells in Proskauer and Beck medium. Right, background uniform growth with clumps of cells interspersed.

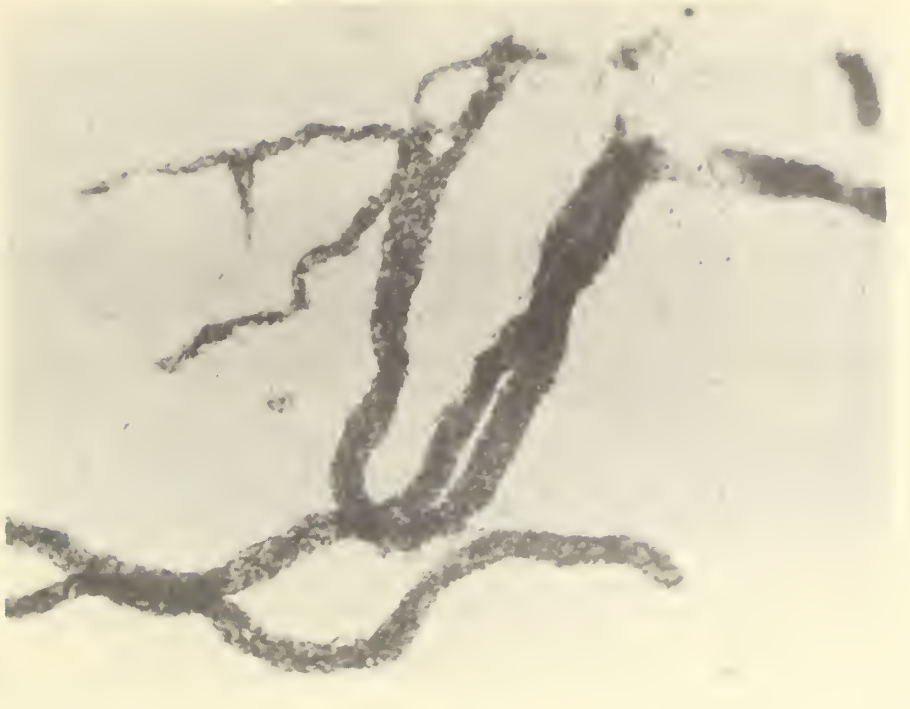

Figure 3.--Cording of mycobacteria.

medium. Thiophen-2-carboxylic acid hydrazide (TCH) (see Appendix C, under Dubos broth with TCH.) is prepared in the same final concentration. Neotetrazolium chloride (see Appendix C, under Dubos broth with INH.) is prepared in a final concentration of one part neotetrazolium to 40,000 parts basal medium.

One tube of each inhibitory agent medium and one tube of basal medium (control) is used to type each isolant. Each tube is inoculated with 0.4 ml. of a Dubos broth culture of the isolant to be typed. Inhibition is indicated when no growth is present in the medium containing the inhibitory agent. The control medium must have luxuriant growth.

Bacterial growth is purple in the neotetrazolium medium because neotetrazolium salts are converted to colored compounds called formazans.

Observations.--M. bovis strains are inhibited by all three agents but growth is luxuriant in the control medium (fig. 4).

M. tuberculosis strains are inhibited by INH and neotetrazolium but grow well in TCH medium and control medium.

M. avium and Runyon's anonymous groups are not inhibited by any of the three agents. Therefore, luxuriant growth occurs in all inhibitory media and in the control medium.

M. bovis and M. tuberculosis cultures are identified on the basis of growth characteristics in Proskauer and Beck medium, the niacin test, drug resistance, and cellular morphology. Animal inoculation is recommended when facilities are available.

M. avium and Runyon's anonymous groups are differentiated by additional observations of culture growth on Lowenstein-Jensen medium. Appearance time, pigmentation, and colonial morphology are observed and recorded.

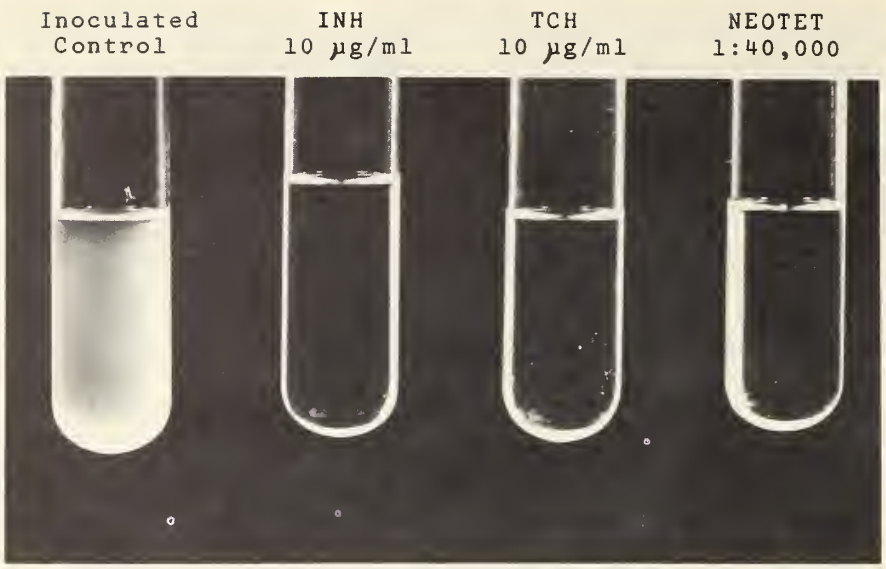

Figure 4.--Inhibition of Mycobacterium bovis by isoniazid, thiophen-2-carboxylic acid hydrazide, and neotetrazolium. (Control tube is on the left.)

Colonies of M. avium and Group III strains appear in 13 to 20 days at 37° C. and in 6 to 13 days at 45° C. Pigmentation is usually absent. The colonies are moist, white, entire, and convex to raised. Final differentiation between these two types is made by animal inoculation (chickens). M. avium causes generalized lesions in the liver, spleen, and kidneys. Group III cultures do not produce lesions in the chicken.

Both Group I and II produce orange to yellow pigment under certain light conditions. Group I colonies are pigmented when grown in light but colorless when grown in darkness. Group II colonies are pigmented when grown in light or in darkness.

The light requirement for the differentiation of Group I and Group II colonies can be satisfied if the culture tubes occupy a position near an incubator door, which is opened several times a day. Satisfactory conditions of darkness can be produced by covering the appropriate culture tubes with aluminum foil.

Group IV microorganisms are characterized by bacterial growth that appear in 6 days or less at 37° C. Temperature requirements for growth are not restrictive. Many strains grow between room temperature and 45° C. Pigment is not usually present in colony growth but may occur occasionally.

Animal Inoculation

The inoculation of laboratory animals may be used as an adjunct to the cultural and biochemical typing techniques presented in this manual. M. tuberculosis, M. bovis, M. avium, and Group III isolants are commonly differentiated by this method. Other Runyon anonymous microorganisms are not typed by animal inoculation because they are not normally virulent for laboratory animals.

The virulence of several mycobacteria for selected laboratory animals is presented in table 1.

Guinea pigs and chickens are each inoculated intraperitoneally with 1.0 ml. of a saline suspension containing 0.1 mg. of bacterial cells per

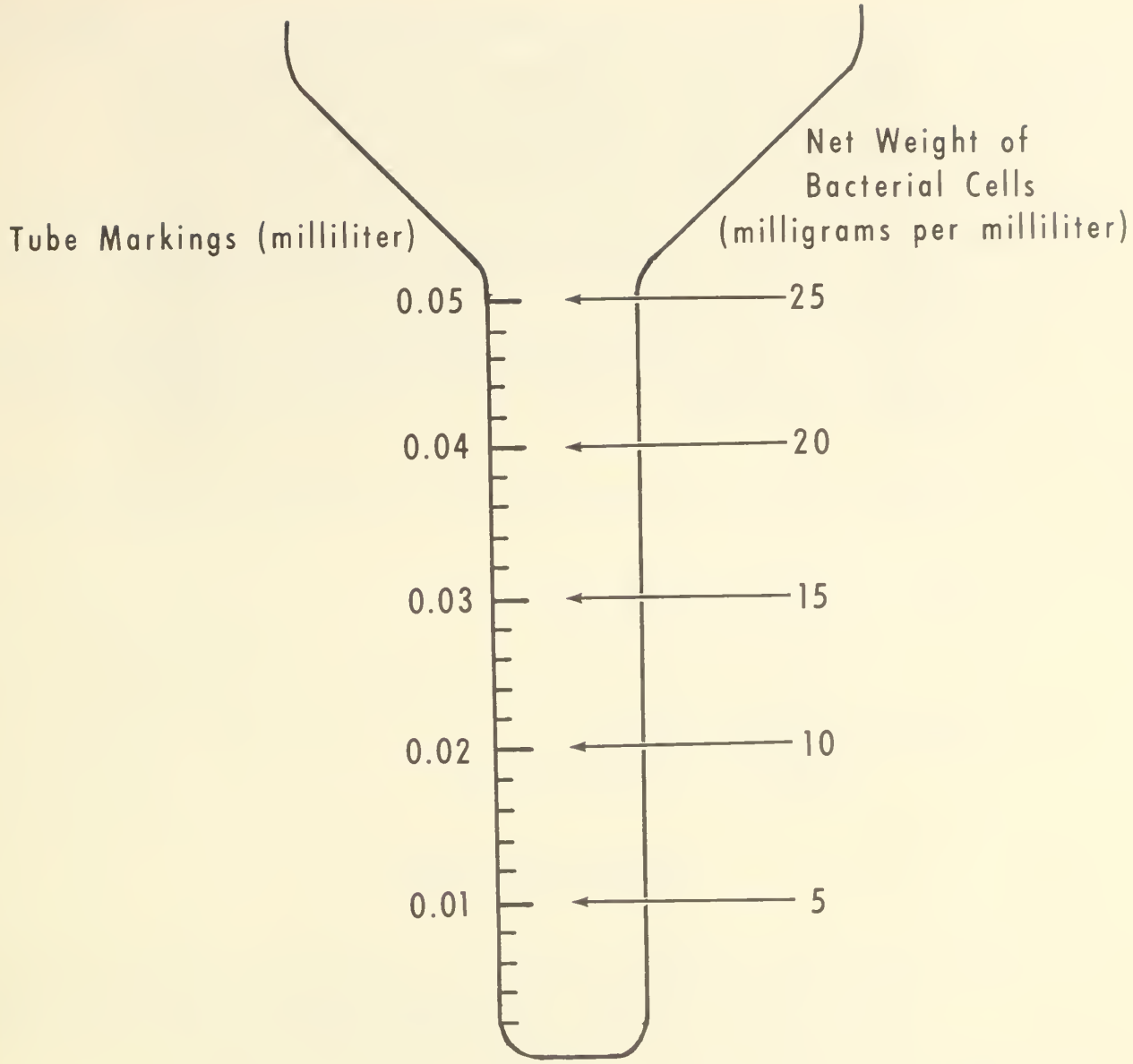

Figure 5.--Translation of Fitch-Hopkins tube readings.

milliliter of saline. Rabbits are inoculated intraperitoneally with 1.0 ml. of saline suspension containing 0.01mg. of bacterial cells per milliliter of saline.

Inoculated animals are kept for 2 months. Any that die within or survive that period are necropsied for evidence of tuberculosis.

The concentration of bacterial suspensions for animal inoculation is determined with the Fitch-Hopkins tube and is centrifuged at 2,357[8] RCF for 20 minutes. The bacterial cells are packed in the bottom of the tube constriction by centrifugal force. The tube calibration adjacent to the top of the packed cell column is used to determine the wet weight of cells in the Dubos broth culture. The translation from Fitch-Hopkins tube caliberations to wet weight of bacterial cells is illustrated in figure 5.

[8] 3,000 r.p.m. with a 9.25 = inch effective radius is sufficient for 2,357 RCF.

The method is explained in the following example:

Needed: 50.0 ml. of a 0.1 mg. per ml. suspension.

Available: 10.0 ml. of visibly turbid Dubos broth culture.

Procedure:

(1) Transfer 2.0 ml. of the Dubos broth culture to a clear Fitch-Hopkins tube.
(2) Centrifuge at 2,357 RCF.
(3) Translate the 0.01 reading on tube constriction to 5.0 mg. cells per ml.
(4) Add 49.0 ml. of sterile physiologic saline solution to 1.0 ml. of the original Dubos broth.
(5) Proof.

Needed: 50 ml. of a 0.1 mg. per ml. of suspension = 5.0 mg. total wet weight of cells.

Add: 49.0 ml. saline solution
1.0 ml. Dubos broth
50.0 ml. total volume of inoculum

Result: 5.0 mg. in 50.0 ml.
Same as: 1.0 mg. in 10.0 ml.
Same as: 0.1 mg. in 1.0 ml.

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APPENDIX A

Safety Measures

I. Health Program for Laboratory Workers

A. Preemployment examination should include a tuberculosis skin test and chest X-ray.
B. People working with mycobacteria should have a tuberculosis skin test every 6 months and a chest X-ray (14 x 17 inches) once a year.
C. People that have a positive reaction to the skin test should not have their skin tested but should be required to take a chest X-ray at the discretion of their physicians.
D. Clerical workers associated with a tuberculosis laboratory should have a skin test and chest X-ray once a year.

II. Procedures in Contaminated Areas

A. Biological safety cabinets (hoods) with a filtered exhaust air system should be used. A negative pressure of 0.75 inch of water is maintained in the cabinet. Material to be removed from the hood is either decontaminated as a batch or individual articles are placed in an airlock at the end of the hood and decontaminated with a suitable disinfectant. A filtered air inlet is desirable to keep contamination to a minimum.
B. Pipettes are used only with a safety pipette bulb. (Materials are never pipetted by mouth.) All discarded pipettes are immersed in a suitable disinfectant. Contaminated pipettes should be sterilized by pressure steam sterilization at 121° C. for 1 hour.
C. Blenders should be used only in a safety cabinet since they can produce considerable aerosol, which is very hazardous.
D. All culture tubes and petri dishes should be opened only in a safety cabinet.
E. Before entering animal rooms where animals are infected with mycobacteria, all clothing should be changed. Rubber gloves should be worn and a surgical mask placed over mouth and nose. An eye shield is used if animals are to be inoculated with live mycobacteria. Upon leaving the animal room, individuals should completely disrobe and shower. Even hair should be washed, unless a head protector is used. Clothes used in the animal room are autoclaved at 121° C. for 45 minutes.
F. Only syringes with permanently attached or lock-on needles are used.

III. Disinfectants

A. Disinfectants used in the laboratory are 2 percent Amphyl (see Appendix D) or 5 percent phenol.
B. Quaternary ammonium compounds should not be used as a tuberculocide.

APPENDIX B

Stain Preparations

Acetic Alcohol

Ingredients		Amount
Alcohol (95 percent)	milliliters	760
Glacial acetic acid	do	40

Alcoholic Eosin Y

Ingredients		Amount
Stock alcoholic eosin solution (1 percent)		
Eosin Y (water and alcohol soluble)	gram	1.0
Distilled water	milliliters	20
Dissolve and add alcohol (95 percent)	do	80
Working eosin solution:		
Eosin (1 percent stock solution)	part	1
Alcohol (80 percent)	parts	3

Add 1 ml. of glacial acetic acid for every 200 ml. of working eosin. Filter before using.

Collodion Solution

Ingredients		Amount
Ether	milliliters	500
Absolute alcohol	do	500
Collodion[1]	do	7.2

[1] See Appendix D.

Harris's Hematoxylin

Ingredients		Amount
Hematoxylin crystals	grams	5
Alcohol (95 percent)	milliliters	50
Aluminum potassium sulfate (alum)	grams	100
Distilled water	milliliters	1,000
Mercuric oxide	grams	2.5

Dissolve the hematoxylin in the alcohol and the alum in the water by the aid of heat. Mix the two solutions. Bring the mixture to a boil as rapidly as possible. Then remove from heat and add mercuric oxide. Reheat solution until it becomes dark purple (about 1 minute), and promptly remove container from flame and plunge it into a basin of cold water. Before using, add 4 ml. of glacial acetic acid to 100 ml. of stock solution.

This stain may be used immediately but gives better results if it is allowed to age for a week. It should be filtered before using.

New Fuchsin Stain

Ingredients		Amount
New Fuchsin[1]	grams	10
Phenol	do	50
Absolute alcohol	milliliters	100

[1] See Appendix D.

Add alcohol slowly, and dissolve solids by shaking.

Working Methylene Blue

Ingredients		Amount
Methylene blue	grams	.5
Glacial acetic acid	milliliters	.5
Tap water	do	100.0

Ziehl-Neelsen Acid-Fast Stain

Ingredients		Amount
Stock saturated alcoholic basic fuchsin solution:		
Ethyl alcohol (95 percent)	milliliters	100
Basic fuchsin	grams	3
Working solution of basic fuchsin:		
Saturated alcoholic basic fuchsin	milliliters	10
Phenol, analytical grade (5 percent)	do	90
Acid alcohol:		
Concentrated hydrochloric acid	do	3.2
Ethyl alcohol (95 percent)	do	97.0
Brilliant green counterstain:		
Brilliant green[1]	gram	1
Sodium hydroxide (0.01 percent)	milliliters	100

[1] See Appendix D.

Fix the smear by heating gently. Stain fixed smear for 3 minutes in steaming Ziehl's carbol fuchsin. (A strip of filter paper laid on the smear tends to conserve reagent and to uniformly distribute stain.) Rinse in distilled water. Decolorize in 95 percent ethyl alcohol, containing 3 percent by volume of concentrated hydrochloric acid, until only a suggestion of pink remains. Wash in distilled water. Counterstain for 3 minutes with alkaline brilliant green. Wash in distilled water and dry. Read with oil-immersion lens of microscope.

APPENDIX C

Culture and Reagent Preparations

Dubos Broth With Tween 80 With Dubos Oleic Albumin Complex With INH

Ingredients	Amount needed per liter
Dubos broth base (Difco 0385)[1] . . . . . grams . .	6.5
Distilled water . . . . . .milliliters . .	890
0.1 percent IHN[1,2] solution. . . . . do	10
Dubos Oleic Albumin Complex (Difco 0375)[1] . . do	100

[1]See Appendix D.

[2]Isonicotinic acid hydrazide; concentration should be 10 μg. per ml. of media.

Preparation.--Mix Dubos broth base and water until in complete solution. Add INH solution and mix thoroughly. Pour into aspirator bottle with attachment for aseptic dispensing. Sterilize in autoclave at 121° C. for 20 minutes. Cool to below 56° C. Add aseptically the Dubos Oleic Albumin Complex and mix well. Dispense aseptically into 20 x 125 mm. screw cap test tubes in 8-ml. amounts. Incubate at 37° C. to check sterility.

Dubos Broth With Tween 80, Dubos Oleic Albumin Complex, and TCH

Ingredients	Amount per liter
Dubos broth base (Difco 0385)[1]. . . . . grams . .	6.5
Distilled water . . . . . milliliters . .	900
Dubos Oleic Albumin Complex (Difco 0375)[1] . . . . . do	100
1 percent solution of TCH[1,2] (filter sterilized). . milliliter . .	1

[1]See Appendix D.

[2]Thiophen-2-carboxylic acid hydrazide, which is used at the rate of 10 mg. per ml. of media.

Preparation.--Mix Dubos broth base and water until in complete solution. Pour into aspirator bottle with attachment for aseptic dispensing. Sterilize in autoclave at 121° C. for 20 minutes. Cool to below 56° C. Add aseptically the Dubos Oleic Albumin Complex and the filter-sterilized TCH solution. Mix well. Dispense aseptically into 20 x 125 mm. screw cap test tubes in 8-ml. amounts. Incubate at 37° C. to check sterility.

Dubos Tween Albumin Broth With Neotetrazolium Chloride (1:40,000)

Ingredients	Amount per liter
Dubos broth base without Tween 80 (Difco 0435)[1] . . grams . .	6.5
Distilled water . . . . . milliliters . .	890
Tween 80 . . . . . milliliter . .	.5
50 percent glucose solution (filter sterilized) . milliliters . .	10
Dubos Oleic Albumin Complex (Difco 0375)[1] . . . . . do	100
1 percent neotetrazolium chloride solution[2] . . . . . do	2.5

[1]See Appendix D.
[2]Autoclaved 15 minutes at 121° C. to sterilize.

Preparation.--Mix and warm Dubos broth base and water until in complete solution. Add Tween 80 to the hot medium and mix thoroughly. Pour into aspirator bottle with attachment for aseptic dispensing. Sterilize in autoclave at 121° C. for 20 minutes. Cool to below 56° C. Add aseptically the sterile glucose solution, Dubos Oleic Albumin Complex, and the neotetrazolium chloride solution. Mix thoroughly. Dispense aseptically into 20 x 125 mm. screw cap test tubes in 8-ml. amounts. Test tubes should be new or absolutely free from scratches so as not to hinder vision of media. Incubate at 37° C. to check sterility.

Herrold's Egg Yolk Medium

Ingredients	Amount needed for 1,020 ml. of media
Peptone (Difco 0118)[1] . . . . . grams . .	9.0
Sodium chloride. . . . . do	4.5
Agar (special Noble-Difco 0142)[1] . . . . . do	15.3
Beef extract (Difco 0126)[1] . . . . . do	2.7
Glycerol. . . . . milliliters . .	27.0
Distilled water . . . . . do	870.0
Egg yolks	6
Malachite green dye solution (2 percent). . . . . milliliters . .	5.1

[1]See Appendix D.

Equipment.--Prepare and sterilize the following: One 2-liter aspirator bottle with cotton gauze plug, a small bell filling attachment for dispensing, preferably a magnetic stirring bar for mixing, rat-tooth forceps for each egg, and 20 x 125 screw cap test tubes in sufficient quantity.

Preparation.--Mix and heat all ingredients (except egg yolks and dye solution) until agar is melted. Cool to 60° C. and adjust pH to 7.5 with 1 N. sodium hydroxide. Pour into aspirator bottle and sterilize in autoclave 25 minutes at 121° C. Cool to 56° C. and add six sterile egg yolks prepared as follows: Use yolks aseptically prepared from strictly fresh eggs. If possible, eggs should be from hens that have had no antibiotics added to their feed or medicine. Scrub eggs with a brush in warm detergent water and rinse with

clear water. Let air-dry on a towel. Soak in 75 percent isopropyl alcohol for 30 minutes. Dry between layers of sterile towels.

Crack shell at one end with sterile forceps, make 10-mm. hole, and remove egg white with forceps and gravity. Make hole larger and break yolk. Twirl forceps to mix yolk, pick out yolk sac with forceps, and pour mixed yolk into sterile media in aspirator bottle. Repeat process for each egg. Mix media gently on magnetic stirrer. Add malachite green dye solution with sterile pipette. Blend again on stirrer.

Dispense aseptically into 20 x 125 mm. screw cap test tubes in 9-ml. amounts. Dispense on bell side of tube only. Do not shake tube after filling to avoid film on more than bell side. Allow medium to harden in slant position. Check sterility by incubating medium for 48 hours at 37° C.

Lowenstein-Jensen Medium

Ingredients	Amount needed for 1,612 ml. of media
Lowenstein medium base (Difco 0444)[1]. . grams . .	37.2
Glycerol[2] . . . . . milliliters . .	12
Distilled water . . . . . do	600
Whole eggs[3] . . . . . do	1,000

[1]See Appendix D.

[2]This medium is also made without glycerol when specified.

[3]Approximately 24 strictly fresh large eggs aseptically prepared. If source is known, eggs should be from hens that have had no antibiotics in feed or medicine.

Equipment.--Prepare and sterilize the following: One 2-liter aspirator bottle with small bell filling attachment for dispensing, and perferably a magnetic stirring bar for mixing. Large funnel lined with two thicknesses of unbleached muslin. Edge of funnel should be taped to hold muslin in place. Huck towel should be folded over muslin-lined funnel and taped in place. Funnel should be wrapped in paper for sterilizing. Two-quart Ball jars with Osterizer blender tops, inverted, and autoclaved. Also, 20 x 125 mm. screw cap test tubes in sufficient quantity. Pipette or glass rod for stirring eggs.

Preparation.--Add Lowenstein medium base to water containing glycerol. Mix on Waring blender to obtain a smooth solution. Heat in flask until dissolved. Pour into a 2-liter aspirator bottle. Sterilize in an autoclave for 20 minutes at 121° C. Cool to room temperature.

Scrub eggs in warm detergent water, rinse in clear water, and air dry on towel. Soak in 75 percent isopropyl alcohol for 30 minutes. Dry eggs between layers of sterile towels. Dip top of beaker in alcohol and flame dry to sterilize. Break eggs on sterile beaker edge and drop in sterile Ball blender jars. Mix on Osterizer just enough to homogenize but not to cause excessive air bubbles.

Filter mixture through sterile muslin-lined funnel into sterile media in aspirator bottle. Put gauze plug from bottle inside towel layer to keep sterile while eggs are filtering. Stir eggs with sterile pipette or rod if necessary to help filtering process, but keep eggs in funnel covered with towel gauze plug in aspirator bottle. Mix gently but thoroughly on magnetic stirrer until media is evenly blended.

Dispense into tubes aseptically. Dispense on bell side of tube only. Do not shake tube after filling to avoid film on more than bell side. Dispense in 9-ml. amounts in 20 x 125 mm. screw cap tubes. Inspissate for 40 minutes at 80° C. with tubes in slant position. Cool slightly. Incubate 24 to 48 hours in slant position so excess moisture will be reabsorbed by media.

Middlebrook's 7H10 Agar With Middlebrook Oleic Acid Albumin, Dextrose and Beef Catalase (OADC) Enrichment

Ingredients	Amount needed per liter
Middlebrook's 7H10 agar base (Difco 0627)[1] . . grams . .	20
Distilled water containing 5 ml. glycerol . milliliters . .	900
Middlebrook OADC enrichment (Difco 0722)[1] . . do	100

[1] See Appendix D.

Mix and heat Middlebrook's 7H10 agar base and water containing glycerol until in complete solution. Pour into aspirator bottle with attachment for aseptic dispensing of flask for pouring plates. Sterilize in autoclave at 121° C. for 15 to 20 minutes. Cool to 56° C., and add Middlebrook OADC enrichment. Mix well. Dispense in 20 x 125 mm. screw cap test tubes in 9-ml. amounts, and allow to solidify in slant position. For plates, pour medium into petri dishes in 25-ml. amounts. Incubate at 37° C. to check sterility.

Modified Proskauer and Beck Medium With 5 Percent Horse Serum

Ingredients	Amount needed per liter
L-asparagine . . . . . .grams . .	5
Potassium phosphate monobasic . . . . . do	5
Potassium sulfate . . . . . do	5
Glycerol. . . . . .milliliters . .	20
Distilled water . . . . . do	930
Magnesium citrate . . . . . .grams . .	1.5
Sterile horse serum . . . . . .milliliters . .	50

Preparation.--Dissolve L-Asparagine by heating in part of the water until clear. Dissolve each of the next two chemicals in small amounts of water separately. Add to asparagine mixture, then add glycerol and rest of water. Mix thoroughly. Adjust pH to 7.0 with 10 N. sodium hydroxide. Now add the 1.5 g. of magnesium citrate. Mix until in solution. Pour into aspirator bottle with attachment for aseptic dispensing. Sterilize in autoclave at 121° C. for 20 minutes. Cool to 50° C., and add aseptically the sterile horse serum. Dispense aseptically into 20 x 125 mm. screw cap test tubes in 4-ml. amounts. Incubate at 37° C. to check sterility.

Niacin Test Reagents

Ingredients	Amount
Cyanogen bromide solution:	
Cyanogen bromide . . . . . grams . .	10
Distilled water. . . . . .milliliters . .	90
Ethyl aniline solution:	
Aniline . . . . . do	4
Ethyl alcohol (95 percent) . . . . . do	96

CAUTION: Cyanogen bromide solution is poisonous and should be prepared and used under the fume hood. Both the cyanogen bromide and the ethyl aniline solutions should be stored in brown bottles no more than 4 weeks before being replaced with fresh solutions.

Stonebrinks Medium

Ingredients	Amount needed for 1,200 ml. of total media (140 slants)
Salt mixture:	
Sodium pyruvate (sodium salt-pyruvic acid). grams. .	5
Potassium phosphate monobasic (KH_2PO_4) . do	2
Distilled water . . . . . milliliters. .	300
Sodium phosphate dibasic (Na_2HPO_4) to obtain pH of 6.5	
Dye mixture:	
Crystal violet . . . . . .milligrams. .	100
Malachite green . . . . . do	800
Distilled water . . . . . milliliters. .	100
Egg mixture:	
Whole egg . . . . . milliliters. .	800

Equipment.--One 250-ml. screw cap flask; one 2-liter aspirator bottle with magnetic stirring bar within a small bell filling attachment on rubber hose to dispense aseptically into tubes; a large funnel lined with two layers of unbleached muslin and covered with huck towel (muslin taped in place) then wrapped in paper and taped to sterilize; two 1-quart jars with Osterizer blender tops, sterilized in inverted position; one pipette or glass rod to stir eggs in funnel; and 20 x 125 mm. screw cap test tubes in sufficient quantity.

Preparation.--Mix salt solution until completely dissolved. Adjust pH to 6.5 with sodium phosphate dibasic. Place in aspirator bottle. Mix dye solution until completely dissolved. Place in screw cap flask. Sterilize by autoclaving at 121° C. for 20 minutes. Cool to room temperature.

Wash eggs with brush in warm detergent water, rinse in clear water, and lay on towel to dry. Soak in 75 percent isopropyl alcohol solution for 30 minutes. Dry by inserting between layers of sterile towels. Dip top of a beaker in alcohol. Burn off alcohol with bunsen burner to sterilize.

Break eggs on sterile edge of beaker and drop into sterile blender jars. Mix gently on Osterizer just enough to homogenize egg mixture but not enough to cause air bubbles. Add dye mixture to salt mixture in the aspirator bottle. Filter eggs through sterile muslin-lined funnel into solutions in the aspirator bottle. Put cotton gauze plug from bottle between layers of the huck towel while eggs are filtering through. Stir eggs with sterile pipette or rod if necessary to help filtering process and keep eggs in funnel covered with towel as much as possible.

When egg mixture has completed filtering, replace cotton gauze plug in aspirator bottle and mix gently but thoroughly on magnetic stirrer until the media is evenly blended. Dispense into tubes aseptically. Dispense on bell side of tube only, and do not shake tube after filling to avoid film on more than bell side.

Dispense in 9-ml. amounts in 20 x 125 mm. screw cap test tubes. Inspissate for 40 minutes at 80° C. with tubes in slant position. Cool slightly, Incubate 24 to 48 hours in slant position so excess moisture will be reabsorbed by media.

APPENDIX D

Materials and Manufacturers

1. Agar (special Noble-Difco 0142)--Difco Laboratories, Detroit, Mich. 48201.
2. Amphyl--Lehn & Fink Products Corps., 4934 Lewis Avenue, Toledo, Ohio.
3. Auramine O--Matheson Coleman & Bell, Cincinnati, Ohio.
4. Autotechnicon--The Technicon Co., Chauncey, N.Y.
5. Beef extract (Difco 0126)--(See No. 1.)
6. Basic fuchsin--National Aniline Division, Biological Stains Department, 40 Rector Street, New York, N.Y.
7. Clearing agent--(See No. 4.)
8. Collodion--Mallinkrodt Chemical Works, St. Louis, New York, Montreal.
9. Decal--Omega Chemical, Cold Spring on Hudson, N.Y. (Distributed by Scientific Products, Division of American Hospital Supply Corp., Evanston, Ill.)
10. Dehydrant--(See No. 4.)
11. Dubos broth base--(See No. 1.)
12. Dubos Oleic Albumin Complex--(See No. 1.)
13. Lab Tek Thermoelectric Centre--Lab Tek Instruments Co., Westmont, Ill.
14. Lowenstein medium base (Difco 0444)--(See No. 1.)
15. Middlebrook OADC enrichment (Difco 0722)--(See No. 1.)
16. Middlebrook's 7H10 agar base (Difco 0627)--(See No. 1.)
17. New Fuchsin--Hartman Leddon Co., Philadelphia, Pa.
18. Paraplast tissue embedding media--Aloe Scientific Division of Brunswick, St. Louis, Mo.
19. Peptone (Difco 0118)--(See No. 1.)
20. Permount--Fischer Scientific Co., Fairlawn, N.J.
21. RDO--DuPage Kinetic Labs, Inc., Box 215, Downers Grove, Ill.
22. Styron 666--Dow Chemical Co., Midland, Mich.
23. TCH--Aldrich Chemical Co., Inc., 2371 North 30th Street, Milwaukee, Wisc.
24. Tissue Tek embedding rings--(See No. 13.)
25. Tween 80--(See No. 1.)

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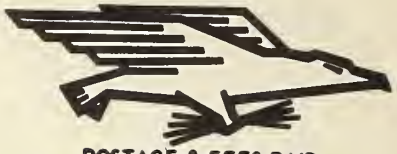

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